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(57) Abstract

The present invention provides primers that allow simultaneous amplification of multiple DNA target sequences present in a DNA sample. Further provided are methods for detecting multiple defined target DNA sequences in a DNA sample. Methods for high-throughput genetic screening are also provided. In yet another aspect, the present invention provides single-stranded oligonucleotide DNA primers for amplification of a target DNA sequence in a multiplex polymerase chain reaction.

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UNIVERSAL PRIMER SEQUENCE FOR MULTIPLEX DNA AMPLIFICATION

Field of the Invention

This invention pertains to universal primers having use in amplification of DNA sequences by methods such as polymerase chain reaction (PCR), specifically to primers that allow the simultaneous amplification of a multiplicity of DNA sequences.

Background of the Invention

Polymerase chain reaction (PCR) is a method whereby virtually any DNA sequence can be selectively amplified. The method involves using paired sets of oligonucleotides of predetermined sequence that hybridize to opposite strands of DNA and define the limits of the sequence to be amplified. The oligonucleotides prime multiple sequential rounds of DNA synthesis catalyzed by a thermostable DNA polymerase. Each round of synthesis is typically separated by a melting and re-annealing step, allowing a given DNA sequence to be amplified several hundred-fold in less than an hour (Saiki et al., *Science* 239:487, 1988).

With the rapid advances in mammalian molecular genetics, an ever increasing number of disease genes have been identified. Accordingly, PCR has gained widespread use for the diagnosis of inherited disorders and the susceptibility to disease. Typically, the region of interest is amplified from either genomic DNA or from a source of specific cDNA encoding the cognate gene product. Mutations or polymorphisms are then identified by subjecting the amplified DNA to analytical techniques such

as DNA sequencing, hybridization with allele-specific oligonucleotides (ASOs), oligonucleotide ligation, restriction endonuclease cleavage or single-strand conformational polymorphism (SSCP) analysis.

For the analysis of small genes and transcripts, or genes where the mutant allele or polymorphism is well characterized, amplification of a single defined region of DNA is sometimes sufficient. When analyzing large genes and transcripts or undefined genes, however, multiple individual PCR reactions are often required to identify critical base changes or deletions. Thus, to streamline the analysis of large complex genes, multiplex PCR (i.e., the simultaneous amplification of different target DNA sequences in a single PCR reaction) has been utilized.

The results obtained with multiplex PCR are, however, frequently complicated by artifacts of the amplification procedure. These include "false-negative" results due to reaction failure and "false-positive" results such as the amplification of spurious products, which may be caused by annealing of the primers to sequences which are related to, but distinct from, the true recognition sequences.

For use in multiplex PCR, a primer should be designed so that its predicted hybridization kinetics are similar to those of the other primers used in the same multiplex reaction. While the annealing temperatures and primer concentrations may be calculated to some degree, conditions generally have to be empirically determined for each multiplex reaction. Since the possibility of non-specific priming increases with each additional primer pair, conditions must be modified as necessary as individual primer sets are added. Moreover, artifacts that result from competition for resources (e.g., depletion of primers) are augmented in multiplex PCR, since differences

in the yields of unequally amplified fragments are enhanced with each cycle.

Weighardt et al. (*PCR Meth.App.* 3:77, 1993) describe the use of 5'-tailed oligonucleotides for PCR. However, a key feature of this amplification method involves separate annealing and primer extension reactions for each primer, which is not practical in a multiplex context. Therefore, complete optimization of the reaction conditions for multiplex PCR can become labor intensive and time consuming. Since different multiplex PCRs may each have unique reaction conditions, development of new diagnostic tests can become very costly.

Thus, there is a need in the art for primers that allow multiplex PCR reactions to be designed and carried out without elaborate optimization steps, irrespective of the potentially divergent properties of the different primers used. Furthermore, there is a need in the art for primers that allow multiplex PCR reactions that, under the same reaction conditions, simultaneously produce equivalent amounts of each of many amplification products.

Summary of the Invention

This invention pertains to primers that allow simultaneous amplification of multiple DNA target sequences present in a DNA sample. According to the invention, the DNA sample in a single reaction mixture is contacted with a multiplicity of paired oligonucleotide primers having the structure 5'-XY-3', wherein: X comprises a sequence that does not hybridize to the target sequence; the melting temperature of a hybrid between X and its complement in the absence of other sequences is greater than about 60°C; and Y comprises a sequence contained within or flanking the target sequence or its complement.

Multiple cycles of melting, reannealing, and DNA synthesis (i.e., a PCR reaction) are thereafter performed with the above mentioned DNA sample and invention oligonucleotide primers. Amplified target sequences may then be detected by any method, including, for example, hybridization with allele-specific oligonucleotides, restriction endonuclease cleavage, or single-strand conformational polymorphism (SSCP) analysis.

The invention also encompasses a method for detecting multiple defined target DNA sequences in a DNA sample. This method is carried out by performing the same procedure set forth above, in which the 3' sequence of one primer of each pair comprises a target DNA sequence itself or its complement. The method includes a further step of detecting the amplification products, preferably by gel electrophoresis. In this embodiment, the presence or absence of an amplification product is diagnostic of the presence or absence of the target sequence in the original DNA sample.

In another aspect, the invention encompasses methods for high-throughput genetic screening. The method, which allows the rapid and simultaneous detection of multiple defined target DNA sequences in DNA samples obtained from a multiplicity of individuals, is carried out by simultaneously amplifying many different target sequences from a large number of patient DNA samples, using oligonucleotide primer pairs as above.

In yet another aspect, the present invention provides single-stranded oligonucleotide DNA primers for amplification of a target DNA sequence in a multiplex polymerase chain reaction. The primers have the structure 5'-XY-3', wherein X comprises an invention primer sequence, and Y comprises a sequence contained within or flanking a target sequence or its complement. Typically, Y comprises

a sequence from 17 to 25 bases in length, and the melting temperature of hybrids between the primers and their complements is at least 72°C.

The methods and compositions of the present invention can be applied to the diagnosis of genetic and infectious diseases, gender determination, genetic linkage analysis, and forensic studies.

Brief Description of the Drawings

Figure 1 is a table listing amplicon-specific oligonucleotide primer sequences.

Figure 2 shows agarose gel analysis of primer concentration and annealing temperature for amplification of CFTR exon 21.

Figure 3 shows agarose gel analysis of CFTR 15-plex amplicon organization.

Figure 4 shows gel comparison of chimeric primers with sequence-specific primers for CFTR 15-plex PCR amplification.

Figure 5 shows the results from multiple gene loci amplifications performed in a single PCR thermal cycler under identical reaction conditions and cycling parameters.

Figure 6 shows the results from multiple gene loci amplifications performed in a single PCR thermal cycler under identical reaction conditions and cycling parameters.

Detailed Description of the Invention

Invention methods described herein provide an amplification system for multiplex PCR which is based on the use of chimeric primers tagged on the 5' end with an unrelated 20 nucleotide sequence (UPS). The examples provided below demonstrate that multiple genomic sequences can be co-amplified under identical reaction conditions and cycling parameters with very little optimization of PCR conditions. Using invention compositions and methods, highly specific and efficient amplification of target sequences can be easily and reproducibly achieved by simple adjustment of the individual primer concentrations, with no additional modification of either the reaction components or annealing temperatures.

Invention chimeric primers produced clean visibly detectable PCR profiles over an 8-fold range of template DNA concentrations (see examples). Moreover, the high level of consistency, with respect to the relative band intensities, increases the informativeness and simplifies the interpretation of the results.

The enhanced specificity and efficiency which is conferred by using invention chimeric primers is due to a normalization of the hybridization kinetics. During the early rounds of the PCR, molecules are synthesized which contain the tagged primers at their 5' ends. Therefore, in all subsequent rounds of amplification, the amplicons synthesized will all have identical 20 bp priming sequences with the predicted hybridization kinetics of the universal primer sequence (UPS) tag. Further, by virtue of the extremely GC-rich region at the 5' end of invention UPS sequences, under PCR conditions optimal for the UPS tag, nucleation is progressing in a primer oriented 5' to 3' direction. In addition, each chimeric primer pair has a 3' sequence-specific region with an internal stability profile

(ΔG) for primer duplexing lower than the UPS tag. Therefore, chimeric primers, which have an overall Tm significantly greater than 72° C serve as highly efficient yet stringent recognition sequences for the subsequent rounds of the PCR.

Through normalizing hybridization kinetics, invention compositions and methods have largely eliminated the need to evaluate diverse reaction conditions and cycling parameters. Therefore, the use of invention UPS-tagged primers provides a less costly method in terms of polymerase, labor and time, and should greatly simplify the development of complex multiplex PCRs to be used in new diagnostic tests.

Definitions:

1. "Amplification" of DNA as used herein denotes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. An "amplicon" is a target DNA sequence that is amplified by PCR.

2. "Multiplex PCR" as used herein refers to the simultaneous amplification of multiple DNA targets in a single polymerase chain reaction (PCR) mixture.

3. "High-throughput" denotes the ability to simultaneously process and screen a large number of DNA samples (e.g. in excess of 100 genomic DNAs) in a rapid and economical manner, as well as to simultaneously screen large numbers of different genetic loci within a single DNA sample.

4. "T_m" as used herein, refers to the melting temperature (temperature at which 50% of the oligonucleotide is a duplex) of the oligonucleotide calculated using the nearest-neighbor thermodynamic values

of Breslauer et al. (*Proc. Natl. Acad. Sci. USA* 83:3746-3750, 1986) for DNA and Freier et al.(*Proc. Natl. Acad. Sci. USA* 83:9373-9377, 1986) for RNA.

5. "ΔG" as used herein, refers to the free energy for the oligonucleotide as calculated by the nearest neighbor method of Breslauer et al. (DNA) and Freier et al. (RNA). The free energy is calculated by the formula: G = H - TS; where H is the enthalpy, S is the entropy and T is the temperature. The free energy is a measure of stability, the greater the negative value, the more stable the duplex formed by the oligonucleotide.

The present invention encompasses methods and compositions that allow the efficient and essentially simultaneous amplification of different target DNA sequences in a single polymerase chain reaction (i.e., multiplex PCR). Preferably, equivalent amounts of each amplification product are obtained. The method utilizes novel chimeric oligonucleotide primers that circumvent the technical difficulties associated with multiplex PCR that result in unequal amplification of different target sequences in the same reaction mix.

For example, in a standard PCR reaction employing more than a single pair of oligonucleotide primers, the obligatory imposition of a single set of reaction conditions generally means that one of the primer sets will function more efficiently in priming, causing the target sequence specified by that set of primers to be selectively amplified in the early cycles of amplification. Furthermore, the more efficient primers will also be depleted from the reaction sooner than the less efficient ones, resulting in the increased accumulation of non-specific amplification products in later cycles of amplification. Clearly, these problems are magnified when

it is desired to use multiple primer pairs (>3-4) in a single reaction.

The methods and compositions of the present invention circumvent these problems by imposing a uniformly high degree of specificity on the annealing reactions that occur between different primers present in the mixture and their cognate target sequences in the DNA template. During the early cycles of amplification, products are synthesized that contain the chimeric primers on either end. The chimeric primers then serve as high stringency recognition sequences for subsequent rounds of amplification. This results in normalizing the annealing efficiency of different primers and their cognate target sequences, and thus also normalizes the degree of amplification of different targets.

Primer Design

Multiplex PCR according to present invention utilizes chimeric oligonucleotide primers that include two domains. The 5' "half" of each primer may comprise any sequence between 17 and 25 bases in length that is unrelated to the target DNA, and has the property of forming hybrids with relatively high melting temperatures (e.g., T_ms > 60°C in the absence of other sequences). In some applications, when the target DNA sequence is embedded in a sequence of low complexity (i.e., <108 bp), primers may be used that form hybrids with lower melting temperatures.

In one embodiment, the 5' sequence comprises 5'-GCGGTCCCAAAAGGGTCAGT-3'. This sequence, which is designated as a "universal primer sequence" (UPS), is derived from the bacteriophage vector M13mp18 (Messing J., *Meth. Enzymol.* 101:20, 1983). Alternative embodiments of invention universal primer sequences for the 5' sequence

comprise the following oligonucleotides:

#1: 5'-CGC CAG GGT TTT CCC AGT CA-3'
#2: 5'-CGC CAG GGG GGG CCC AGT CA-3'
#3: 5'-CGG CAG CGG GGC CCA GTC CA-3'
#4: 5'-CGC GGC CGG GGC CCA TCC CA-3'
#5: 5'-CGC GGC CGG GGC CAT CTC AA-3'
#6: 5'-GAG GCC GGT GGC CAT GTC AA-3'
#7: 5'-TAG GCG CGT GGC CAT GTC AA-3'
#8: 5'-TAG GCC CGT GGC CAT GTT AA-3'
#9: 5'-TAG GCC CGT GGC AAT ATT AA-3'
#10: 5'-CCG GTC CGT GGC AAT ATT AA-3'
#11: 5'-CCG GCT CGT GGC GAT GTT AA-3'
#12: 5'-CCG GCG TGT GCC GAT ATT AA-3'
#13: 5'-CCA TGC GTG TGC CGA TAT TA-3'
#14: 5'-CAA TGC GGG CGC CGA TAT TA-3'
#15: 5'-CGA TGC GGG AGC CAA TAT AA-3'
#16: 5'-AGA TGC GGT AGC CAA TAT AA-3'
#17: 5'-GGC GTG CTG AGC CAA TAT GG-3'
#18: 5'-GGC GCG CTG AGC CAA TAT GG-3'
#19: 5'-GGC GCG CCG AGC CAA TAT GG-3'
#20: 5'-GGC GCG CCG AGC TAA TAT AT-3'
#21: 5'-AGC TCG GCG AGC TAA TAT AT-3'
#22: 5'-AGC GCG GCC AGC TAA GAG AT-3'
#23: 5'-CGC GCG GCC GGC TGG AGA GA-3'
#24: 5'-CGC GAG GCC GGC TGT AGA GG-3'
#25: 5'-CGC GAG GCC AGC GGC CGA GG-3'
#26: 5'-CGC GAG GCC AGC GGT CGA GG-3'
#27: 5'-CGC GAG GCC AGC GGT CGA GG-3'
#28: 5'-CGC GGG GCC CGC GGC CGC GG-3'
#29: 5'-CGC CCG CCG CGC CCC GCG CC-3'
#30: 5'-GGC GCT CCA TTA GCG TGA GT-3'

The 3' "half" of each primer comprises a target-specific sequence, i.e., a sequence that is either present or potentially present in the target DNA or its complement.

These 3' sequences may comprise without limitation any such sequence of 17-25 bases, and preferably 20 bases, irrespective of the melting temperatures of hybrids formed between the isolated sequence and its complement.

In one embodiment, the 3' half of the primer is intended to hybridize with a genomic sequence flanking the target sequence of interest; in this case, the primer is used to amplify the target sequence for subsequent diagnostic tests such as, e.g., hybridization with allele-specific oligonucleotides, restriction endonuclease cleavage, or single-strand conformational polymorphism (SSCP) analysis. For this purpose, the 3' half of the primer must correspond to a sequence known to be present in all DNA samples to be tested (or its complement). Non-limiting examples of 3' primer halves useful in practicing the present invention are shown in Figure 1.

In another embodiment, the amplification reaction itself serves as the critical diagnostic step. In this case, the 3' sequence of the primer corresponds to a defined wild-type version of a particular amplicon or its complement (or to a variant version or its complement) whose presence or absence is being tested. When such allele-specific sequences are incorporated into chimeric PCR primers according to the present invention, and the chimeric primers are used in amplification reactions, the absence of a given amplification product is considered definitive for the absence of the allele in the DNA sample being tested.

For use in a given multiplex PCR reaction, target-specific primer sequences are typically analyzed as a group to evaluate the potential for fortuitous dimer formation between different primers. This evaluation may be achieved using commercially available computer programs for sequence analysis, such as Gene Runner, Hastings

Software Inc. Other variables, such as the preferred concentrations of Mg^{++}, dNTPs, polymerase, and primers, are optimized using methods well-known in the art (Edwards et al., *PCR Meth. App.* 3:565,1994).

DNA templates

Any DNA sample may be used in practicing the present invention, including without limitation eukaryotic, prokaryotic and viral DNA. In a preferred embodiment, the target DNA represents a sample of genomic DNA isolated from a patient. This DNA may be obtained from any cell source or body fluid. Non-limiting examples of cell sources available in clinical practice include blood cells, buccal cells, cervicovaginal cells, epithelial cells from urine, fetal cells, or any cells present in tissue obtained by biopsy. Body fluids include blood, urine, cerebrospinal fluid, semen and tissue exudates at the site of infection or inflammation. DNA is extracted from the cell source or body fluid using any of the numerous methods that are standard in the art. It will be understood that the particular method used to extract DNA will depend on the nature of the source. The preferred amount of DNA to be extracted for use in the present invention is at least 5 pg (corresponding to about 1 cell equivalent of a genome size of 4×10^9 base pairs).

Multiplex PCR reaction conditions

In practicing the present invention, a DNA sample is contacted with pairs of chimeric oligonucleotide primers under conditions suitable for polymerase chain reaction. Standard PCR reaction conditions may be used, e.g., 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 µM deoxynucleotide triphosphates (dNTPs), and 25-100 U/ml Taq polymerase (Perkin-Elmer, Norwalk, CT).

The concentration of each chimeric primer in the reaction mixture can range from about 0.05 to about 4 μM. The optimal concentration for primer is evaluated by performing single PCR reactions using each primer pair individually. Similarly, each primer pair is evaluated independently to confirm that all primer pairs to be included in a single multiplex PCR reaction require the same amplification conditions (i.e., temperature, duration of annealing and extension steps). It was found (see example below) that all chimeric primers containing the M13 derived UPS as the 5' half of their sequence could be used at a broad range of annealing temperatures (i.e., 50-60°C).

Multiplex PCR reactions are carried out using manual or automatic thermal cycling. Any commercially available thermal cycler may be used, such as, e.g., Perkin-Elmer 9600 cycler.

Finally, the reaction products are analyzed using any of several methods that are well-known in the art. Preferably, agarose gel electrophoresis is used to rapidly resolve and identify each of the amplified sequences. In a multiplex reaction, different amplified sequences are preferably of distinct sizes and thus can be resolved in a single gel. In one embodiment, the reaction mixture is treated with one or more restriction endonucleases prior to electrophoresis. Alternative methods of product analysis include without limitation dot-blot hybridization with allele-specific oligonucleotides and SSCP.

The following examples are intended to further illustrate the present invention without limiting the invention thereof.

Example 1: Primer design:

Sequence-specific primers were chosen without regard to hairpin formation and having a calculated ΔG for primer duplexing below -10 kcal/mole. The T_m of these primers range from 52° to 68° C as determined by the A+T/G+C method. To evaluate potential primer dimer formation within a primer set, primers sets were analyzed using Amplify 1.2 software (University of Wisconsin, Department of Genetics, Madison, WI). The universal primer sequence (UPS) 5' GCGGTCCCAAAAGGGTCAGT 3' is from bacteriophage M13mp18.

The UPS-tagged primers contain the 20 nucleotide UPS sequence attached to the 5' end of the individual sequence-specific primers listed in Figure 1.

Oligonucleotide primers were synthesized by Operon Technologies (Alameda, CA). Oligonucleotides were HPLC purified and quantitated by spectrophotometry.

Example 2: DNA preparation:

Whole blood samples were collected in high glucose ACD Vacutainers™ (Beckton Dickenson & Co., Franklin Lanes, NJ). Following centrifugation, the buffy coat was collected and lysed with two washes of a 10:1 (v/v) solution of 14 mM NH_4Cl and 1 mM $NaHCO_3$. The lymphocytes were harvested by centrifugation, resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 2 mM EDTA, 0.5% SDS, 500 µg/ml proteinase K) and incubated overnight at 37°C. Samples were then extracted with 1/4th volume of saturated NaCl, and the DNA was collected by ethanol precipitation. The final DNA pellet was washed

with 70% ethanol, air dried and dissolved in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

Buccal cell samples were obtained by brushing the lining of the buccal cavity for 30 seconds with a sterile cytology brush (Scientific Products #S7766-1a). DNA was prepared by immersing the brushes in 600 µl of 50 mM NaOH in 1.2 ml 96-well polypropylene tubes (USA/Scientific Plastics, Ocala, Florida) and vortexed. The tubes, still containing the brushes, were heated to 95° C for 5 min. and the brushes were carefully removed. The lysates were neutralized with 60 µl of 1 M Tris-HCl (pH 8.0) and vortexed. Samples were stored at 4° C.

Example 3: Amplification reactions:

Single amplifications were performed using 4 µl (1-2µg) and 10 µl (5-50ng) of genomic DNA prepared (described above) from either blood or buccal cells, respectively. 50 µl reaction mixtures were carried out in 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM dNTPs, and 2.5 units Taq polymerase (Perkin-Elmer, Norwalk, CT).

Multiplex PCR reactions were carried in a volume of 100 µl under the same conditions, except that 10 units of Taq polymerase per reaction was used.

To demonstrate the use of tagged primers for multiplex PCR, the 32 primer pairs presented in Figure 1 were employed either alone, or as chimeric primers containing a 20 nucleotide tag (GCGGTCCCAAAAGGGTCAGT) corresponding to the M13mp18 universal primer sequence (UPS) at the 5' end. The T_m for all of the chimeric primers was calculated to be greater than 72° C.

Primer concentrations ranged from 0.25 to 1.0 μM. Amplifications were carried out using a Perkin-Elmer 9600 themocycler (Perkin-Elmer, Norwalk, CT) for 28 cycles with ramping (melting at 94°C for 10s , annealing at 50°C, 55°C, 60°C, or 65°C for 10 s, and extension at 72°C for 10s).

After completion of the reaction, 8 μl of the reaction products were loaded directly onto a 2% ethidium bromide-stained agarose gel and subjected to electrophoresis at 250 volts for 90 minutes. The amplification products were visualized with a UV transilluminator and photographed with an Alpha Innotech IS-500 Digital Imaging System version 1.97 (Sun BIO Science, Inc., Branford, CT).

The following experiments were performed to demonstrate the effects on amplification of incorporating invention primer sequences into PCR primers.

Example 4: Effect of Chimeric Primers on Efficiency and Specificity of Amplification

Three sequence-specific primer pairs used to amplify Exon 21 of the cystic fibrosis transmembrane regulator (CFTR) gene (Kerem et al., *Science* 245:1073, 1989) are shown in Figure 2. A chimeric version of one of the three primers was synthesized containing the M13 UPS sequence 5'-GCGGTCCCAAAAGGGTCAGT-3' immediately 5' to the illustrated sequences. The oligonucleotides were synthesized using conventional chemistry and were purified by high-performance liquid chromatography prior to use.

Primers were serially diluted two-fold for each primer pair (SS#1, SS#2, SS#3, and SS#3+UPS. See Figure 1). Primer pairs SS#1, SS#2 and SS#3 are sequence specific primers only; SS#3 + UPS is chimeric with the 5' UPS sequence.

The results show that the addition of the UPS tag confers enhanced specificity over a four fold range of primer concentrations while retaining amplification efficiency at annealing temperatures from 50° to 65° C. Based on this and similar analyses of additional chimeric primer pairs, an annealing temperature of 60° C was determined to be optimal for PCR amplification using UPS-tagged primers.

The efficiencies with which the three CFTR primer pairs (designated SS#1, SS#2, and SS#3) primed amplification varied with primer concentration and temperature of annealing (Figure 2). The primer concentrations were as follows: Lanes 1, 4, 7, and 10, (1.0 μM); lanes 2, 5, 8, and 11, (0.5 μM); and lanes 3, 6, 9, and 12, (0.25 μM). The temperatures of annealing were 50°C, 55°C, 60°C and 65°C, as indicated.

The SS#1 and SS#3 primers, for example, were noticeably inefficient at annealing temperatures above 60°C. The primer pair designated SS#3-UPS, which corresponds to the SS#3 primers having the M13 UPS sequence on their 5' termini, was highly efficient in priming at all temperatures tested; furthermore, few spurious amplification products were detected in reactions containing SS#3-UPS primers. By contrast, SS#2 primers gave spurious amplification products at all three temperatures below 65°C.

Example 5: Multiplex Amplification with Chimeric Primers

To assess the use of chimeric primers for multiplex PCR, a system was designed using 15 UPS-tagged primer pairs specific for the cystic fibrosis transmembrane conductance regulator locus (*CFTR*). An example of the gel

electrophoretic pattern of the multiplex PCR products is shown in Figure 3.

As demonstrated for exon 21 (Figure 2), each chimeric primer pair concentration used within the CFTR 15-plex was determined by performing independent amplicon amplifications over a range of concentrations. Lanes 1-3; multiplex amplification of 3 different genomic DNA samples isolated from blood. All samples were amplified with CFTR chimeric 15-plex primers (See Figure 1) at the following primer pair concentrations: Int 19; 0.5μM. exon 19; 0.5μM. exon 21; 1.0μM. exon 9; 0.75μM. exon 13; 1.0μM. exon 4; 1.0μM. exon 17b; 0.5μM. exon 7; 0.5μM. exon 11; 1.0μM. exon 10; 1.0μM. exon 20; 0.25μM. exon 5; 0.5μM. exon 14b; 0.5μM. exon 12; 0.5μM. exon 3; 0.25μM. Lane M; ΦX174 Hae III digested marker DNA.

Using the same amplification conditions that were originally defined for the individual chimeric primer pairs, all 15 of the predicted *CFTR* PCR products co-amplified with relative ease. It should be emphasized that the *CFTR* multiplex reactions presented in Figure 3 and all subsequent reactions employed the same reaction components, cycling times and temperatures without modification from the single amplicon assay conditions. The only optimization required was an adjustment of the primer concentrations for intron 19 and exon 19 to generate comparable band intensities of the co-amplified products.

Example 6: Comparison of Multiplex PCR Reactions Using CFTR Primer Pairs Lacking and Containing M13 UPS

To demonstrate the enhanced specificity and efficiency conferred by the UPS sequence in multiplex PCR, parallel reactions were carried out using UPS-tagged *CFTR*

primers and the corresponding non-tagged primers. Identical reaction conditions, cycling times and primer concentrations were used for both primer sets. Previously, optimal primer concentrations were determined for the individual primer pairs. Coincidentally, the optimal concentrations determined in this manner were identical for both the UPS and non-UPS tagged primers.

Figure 4 shows the results of agarose gel comparison of chimeric primers with sequence-specific primers for CFTR 15-plex PCR amplification. Lanes 1-8; genomic DNA samples isolated from blood. Lanes 9-16; genomic DNA samples isolated from buccal cells. Each consecutive sample in the group of four amplifications represents a two-fold serial dilution of genomic DNA. Lanes 1-4 and 9-12; CFTR 15-plex amplification with chimeric primers. Lanes 5-8 and 13-16; CFTR 15-plex amplification with sequence-specific primers. Lane M; ΦX174 Hae III digested marker DNA.

As shown, the multiplex amplification using the standard sequence-specific primer pairs failed to generate a clear multiplex PCR profile of the *CFTR* locus. Specifically, several of the expected bands were clearly under-represented, presumably due to differential PCR amplification of their respective products (Figure 4, lanes 5-8 and lanes 13-16).

In contrast, a clear multiplex profile was obtained when the *CFTR* locus was amplified with the corresponding UPS-tagged primer pairs. The expected bands were clearly prominent and virtually free of contaminating products (Figure 4, lanes 1-4 and 9-12). Moreover, equivalent banding patterns were observed over an 8-fold range of template concentrations when UPS-tagged primer pairs were employed.

Conversely, the amplification profile generated using sequence-specific primers was sensitive to variations in the template concentration as evident by the changes in the intensity of individual bands (Figure 4, lanes 5-8 and 13-16).

Example 7: Use of Multiplex PCR to Simultaneously Amplify Different Disease-related Sequences Under Identical Conditions

To further demonstrate the general utility of the UPS-tagged primers, 29 different human genomic target sequences (Figure 1) were amplified in a single thermal cycler under identical reaction conditions and cycling parameters. Results from these amplification reactions using sequence-specific primer pairs and the cognate UPS-tagged primer pairs are presented in Figure 5 and Figure 6.

The banding patterns for the following primer sets are displayed in Figure 5: lanes 1-3 amplification of CFTR 15-plex with chimeric primers; lanes 4-6 amplification of CFTR 15-plex with sequence-specific primers; lanes 7-9 multiplex amplification of α-galactosidase Gaucher's disease (GCR)(Kornreich et al., *Nuc. Acids Res.* 17:3301, 1989) 3-plex and single Sickle Cell Anemia (SCA)(Navon et al., *Science* 243:1471, 1989) target with chimeric primers; lanes 10-12 multiplex amplification of GCR 3-plex and single SCA target with sequence specific primers; lanes 13-15 multiplex amplification of single GCR target and Tay-Sachs (TS) (Tanaka et al., *Am. J. Hum. Genet.* 46:329, 1990) 2-plex with chimeric primers; lanes 16-18 multiplex amplification of single GCR target and TS 2-plex with sequence-specific primers; lanes 19-21 amplification of single ß-thalassemia target with chimeric primers; and

lanes 22-24 amplification of single ß-thalassemia target with sequence-specific primers. Lane M; ΦX174 Hae III digested marker DNA. All primer pairs were tested on 3 different genomic DNA samples isolated from: lanes 1-8 genomic DNA samples isolated from blood cells, while in lanes 9-12 the genomic DNA template was derived from buccal cells.

Figure 6 shows the results of independent amplification of six target sequences within the human Wilms Tumor gene (WT1) (Varanasi et al., *Proc. Natl. Acad. Sci. USA* 91:3554, 1994). Lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21 and 22; amplifications performed with chimeric primers. Lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23 and 24; amplifications performed with sequence-specific primers. Amplifications represent each of six amplicons within the WT-1 gene (B, F, II, J, N, and O; see Figure 1). All primer pairs tested on two independent genomic DNA samples isolated from blood. Lane M; ΦX174 Hae III digested marker DNA. With the exception of one primer pair (Figure 6, lanes 5-8) which did not generate a detectable product when the chimeric primer was employed, the presence of the UPS sequence enhanced the yield of the respective PCR products (Figure 6, lanes 1-4, 9-12, 13-16, 17-20, 21-24).

Results from the multiplex PCR reactions presented herein demonstrate that for both single gene multiplexes (Figure 5, lanes 1-3) and multiplex reactions involving more than one gene (Figure 5, lanes 7-9, 13-15), the UPS-tagged primers generated only the desired bands, and the co-amplified products were more uniform with respect to the band intensities than the corresponding products generated from the non-tagged sequence-specific primers (Figure 6, lanes 4-6, 10-12, 16-18).

What is Claimed is:

1. A single-stranded oligonucleotide DNA primer for amplification of a target DNA sequence capable of use in a multiplex polymerase chain reaction (PCR), said primer having the structure 5'-XY-3', wherein

a) X comprises a sequence that does not hybridize to said target sequence;

b) the melting temperature of a hybrid between X and its complement in the absence of other sequences is greater than about 60°C; and

c) Y comprises a sequence contained within or flanking said target sequence or its complement.

2. The primer of claim 1, wherein X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3'.

3. The primer of claim 1, wherein X comprises the sequence 5'-CGCCAGGGTTTTCCCAGTCA-3'.

4. The primer of claim 1, wherein X comprises the sequence 5'-CGCCAGGGGGGGCCCAGTCA-3'.

5. The primer of claim 1, wherein X comprises the sequence 5'-CGGCAGCGGGGCCCAGTCCA-3'.

6. The primer of claim 1, wherein X comprises the sequence 5'-CGCGGCCGGGGCCCATCCCA-3'.

7. The primer of claim 1, wherein X comprises the sequence 5'-CGCGGCCGGGGCCATCTCAA-3'.

8. The primer of claim 1, wherein X comprises the sequence 5'-GAGGCCGGTGGCCATGTCAA-3'.

9. The primer of claim 1, wherein X comprises the sequence 5'-TAGGCGCGTGGCCATGTCAA-3'.

10. The primer of claim 1, wherein X comprises the sequence 5'-TAGGCCCGTGGCCATGTTAA-3'.

11. The primer of claim 1, wherein X comprises the sequence 5'-TAGGCCCGTGGCAATATTAA-3'.

12. The primer of claim 1, wherein X comprises the sequence 5'-CCGGTCCGTGGCAATATTAA-3'.

13. The primer of claim 1, wherein X comprises the sequence 5'-CCGGCTCGTGGCGATGTTAA-3'.

14. The primer of claim 1, wherein X comprises the sequence 5'-CCGGCGTGTGCCGATATTAA-3'.

15. The primer of claim 1, wherein X comprises the sequence 5'-CCATGCGTGTGCCGATATTA-3'.

16. The primer of claim 1, wherein X comprises the sequence 5'-CAATGCGGGCGCCGATATTA-3'.

17. The primer of claim 1, wherein X comprises the sequence 5'-CGATGCGGGAGCCAATATAA-3'.

18. The primer of claim 1, wherein X comprises the sequence 5'-AGATGCGGTAGCCAATATAA-3'.

19. The primer of claim 1, wherein X comprises the sequence 5'-GGCGTGCTGAGCCAATATGG-3'.

20. The primer of claim 1, wherein X comprises the sequence 5'-GGCGCGCTGAGCCAATATGG-3'.

21. The primer of claim 1, wherein X comprises the sequence: 5'-GGCGCGCCGAGCCAATATGG-3'.

22. The primer of claim 1, wherein X comprises the sequence 5'-GGCGCGCCGAGCTAATATAT-3'.

23. The primer of claim 1, wherein X comprises the sequence 5'-AGCTCGGCGAGCTAATATAT-3'.

24. The primer of claim 1, wherein X comprises the sequence 5'-AGCGCGGCCAGCTAAGAGAT-3'.

25. The primer of claim 1, wherein X comprises the sequence 5'-CGCGCGGCCGGCTGGAGAGA-3'.

26. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCGGCTGTAGAGG-3'.

27. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCAGCGGCCGAGG-3'.

28. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCAGCGGTCGAGG-3'.

29. The primer of claim 1, wherein X comprises the sequence 5'-CGCGAGGCCAGCGGTCGAGG-3'.

30. The primer of claim 1, wherein X comprises the sequence 5'-CGCGGGGCCCGCGGCCGCGG-3'.

31. The primer of claim 1, wherein X comprises the sequence 5'-CGCCCGCCGCGCCCCGCGCC-3'.

32. The primer of claim 1, wherein X comprises the sequence 5'-GGCGCTCCATTAGCGTGAGT-3'.

33. The primer of claim 1, wherein X and Y each comprise from 17 to 20 bases.

34. The primer of claim 1, wherein the melting temperature of a hybrid formed between said primer and its complement in a solution of 0.5M NaCl is at least 72°C.

35. An oligonucleotide DNA primer for amplification of a target DNA sequence, wherein said primer consists of the sequence 5'-GCGGTCCCAAAAGGGTCAGT[Y]-3', wherein Y comprises a sequence contained within or flanking said target sequence or its complement.

36. A method for simultaneous amplification of multiple DNA target sequences present in a DNA sample, which comprises:

a) contacting said DNA sample in a single reaction mixture with a multiplicity of paired oligonucleotide primers having the structure 5'-XY-3', wherein

(i) X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3', and

(ii) Y comprises a sequence contained within or flanking said target sequence or its complement; and

b) performing multiple cycles of melting, reannealing, and DNA synthesis.

37. A method for detecting multiple defined target DNA sequences in a DNA sample, which comprises the steps of:

a) contacting said DNA sample in a single reaction mixture with a multiplicity of oligonucleotide pairs, each of said pairs consisting of a first and second oligonucleotide primer, wherein

(i) said first primer of each pair has the structure 5'-XY-3', wherein X comprises the

sequence 5'-GCGGTCCCAAAAGGGTCAGT-3' and Y comprises a sequence contained within the target sequence or its complement, and

(ii) said second primer of each pair has the structure 5'-XY-3', wherein X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3', and Y comprises a sequence flanking the target sequence or its complement;

b) performing multiple cycles of melting, re-annealing, and DNA synthesis to form amplification products of DNA samples primed with said oligonucleotides; and

c) detecting the amplification products.

38. The method of claim 37 wherein detection of an amplification product indicates the presence of the target sequence in the DNA sample.

39. The method of claim 37 wherein said detecting step comprises gel electrophoresis.

40. A method for high-throughput genetic screening to simultaneously detect the presence of multiple defined target DNA sequences in DNA samples obtained from a multiplicity of individuals, said method comprising the steps of:

a) providing a sample of DNA from each of said individuals;

b) simultaneously contacting each of said DNA samples obtained in a) with a multiplicity of oligonucleotide pairs, each of said pairs consisting of a first and second oligonucleotide primer, wherein

(i) said first primer of each pair has the structure 5'-XY-3', wherein X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3' and Y comprises a sequence contained within the target sequence or its complement, and

(ii) said second primer of each pair has the structure 5'-XY-3', wherein X comprises the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3', and Y comprises a sequence flanking the target sequence or its complement;

c) performing multiple cycles of melting, re-annealing, and DNA synthesis to form amplification products; and

d) detecting the amplification products.

41. The method of claim 40 wherein detection of an amplification product indicates the presence of the target sequence in the DNA sample.

42. The method of claim 40 wherein said detecting step comprise gel electrophoresis.

Cystic Fibrosis Transmembrane Regulator(CFTR) 15-plex

Primer Sequences	Exon	Size (bp)
AGG CTT CTC AGT GAT CTG TTG GAA TCA TTC AGT GGG TAT AAG CAG	Int 19	~440
GCC CGA CAA ATA ACC AAG TGA AGT CTA ACA AAG CAA GCA GTG	19	410
TGA TGG TAA GTA CAT GGG TG CAA AAG TAC CTG TTG CTC CA	21	381
CTT CTA ATG GTG ATG ACA GCC T CCA CTG AAA ATA ATA TGA GGA AAT	9	335
AGG TAG CAG CTA TTT TTA TGG TAA GGG AGT CTT TTG CAC AA	13	295
TGT AGG AAG TCA CCA AAG CGA TAC AGA ATA TAT GTG CC	4	267
GGA GTC CAA TTT TCA CTC ATC TTG AGT TAA TGA GTT CAT AGT ACC TGT T	17b	245
AGA TAC TTC AAT AGC TCA GCC GGT ACA TTA CCT GTA TTT TGT TT	7	220
CAG ATT GAG CAT ACT AAA AGT G TAC ATG AAT GAC ATT TAC AGC A	11	200
GAG CCT TCA GAG GGT AAA AT	10	175

Gauchers(GCR) and Sickle Cell Anemia(SCA) 4-plex

GCR Primer Sequences	Exon	Size (bp)
GGG TGG GAG GGT GGA GGC TAA TGG CCA GAA GGT AGA AAG GTG AG	6	401
GAA TGT CCC AAG CCT TTG A AAG CTG AAG CAA GAG AAT CG	2	358
TGC AAC TAC TGA GGC ACT T TAC AAT GAT GGG ACT GTC G	9	319
SCA Primer Sequences		
CAT TTG CTT CTG ACA CAA CTG CCA ACT TCA TCC ACG TTC ACC		124

GCR and Tay-Sachs (TS) 3-plex

GCR		
CCT TGC CCT GAA CCC CGA A CTG ACT CTG TCC CTT TAA TGC CCA	9, 10, 11	871
TS Primer Sequences		
GTG TGG CGA GAG GAT ATT CCA TGG CTA GAT GGG ATT GGG TCT	11, 12***	530
GGG TCC TAC AAC CCT GTC ACC CAC AAG CTT CAC TCT GAG CAT AAC AAG	7***	190
B-thalassemia Primer Sequences		
GCT GTC ATC ACT TAG ACC TC GCA AGA AAG CGA GCT TAG TG	1, 2, 3	1612

FIG. IA

	Exon	Size (bp)
AAG AAC TGG ATC AGG GAA GA TCC TTT TGC TCA CCT GTG GT	20	155
GCT GTC AAG CCG TGT TCT A GTA TAA TTT ATA ACA ATA GTG CC	5	132
TTG GTT GTG CTG TGG CTC CT ACA ATA CAT ACA AAC ATA GTG G	14b	110
GAC TCT CCT TTT GGA TAC CTA GCA TGA GCA TTA TAA GTA AGG	12	90
GGC GAT GTT TTT TCT GGA GA ACA AAT GAG ATC CTT ACC CC	3	70

CFTR Exon 21 Primer Sequences	Name	Size (bp)
CAA GTG AAT CCT GAG CGT GAT TT CAA AAG TAC CTG TTG CTC CA	SS#1	477
GAA CTT GAT GGT AAG TAC ATG GGT G AGT CAA AAG TAC CTG TTG CTC CAG	SS#2	389
TGA TGG TAA GTA CAT GGG TG GAA AAG TAC CTG TTG CTC CA	SS#3	381

WT-1 Primer Sequences	Name	Size (bp)
CTG AGT GAA TGG AGC GGC GGG TGA ATG AGT AGG TGG	B*	204
CGG TGC TGG ACT TTG CG AAG TGG ACA GTG AAG GCG	F	186
CCG TCT TGC GAG AGC ACC CTA ATT TGC TGT GGG TTA GG	H*	262
AGT TGT GTA TAT TTG TGG TTA TG GTT ACT GTG GAA AGG CAA TG	J	167
GAG ATC CCC TTT TCC AG CAC AGC TGC CAG CAA TG	N*	176
CTC ACT GTG CCC ACA TTG CAA TTT CAT TCC ACA ATA G	O*	211

* Reported previously by Varanasi et al 1994.
** Reported previously by Navon & Proia 1989.
***Reported previously by Tanaka et al 1990.

NOTE:
Amplicon sizes Increase by 40bp for chimeric primers.

FIG. IB

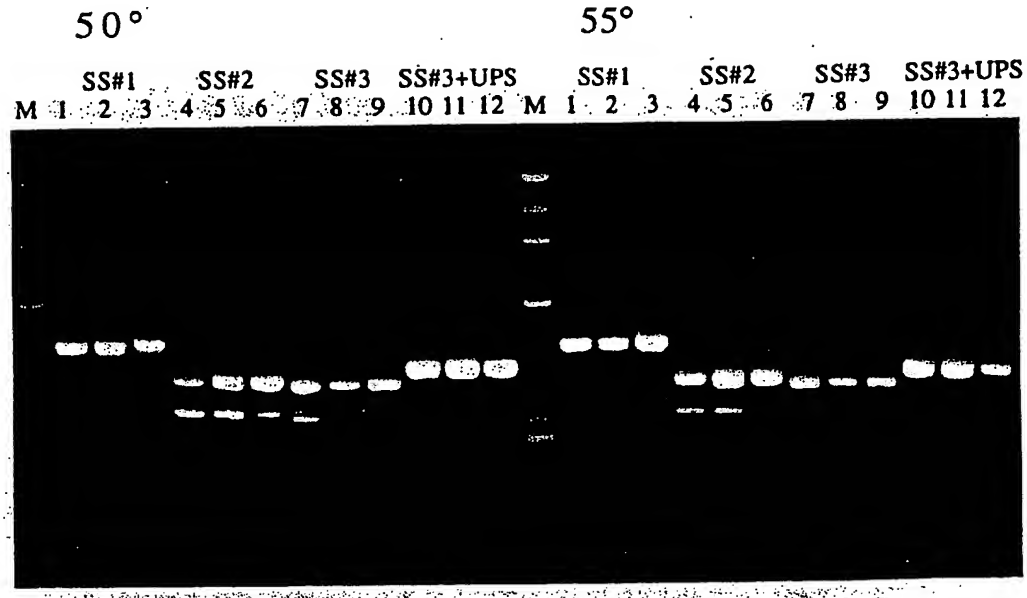

FIG. 2A

FIG. 2B

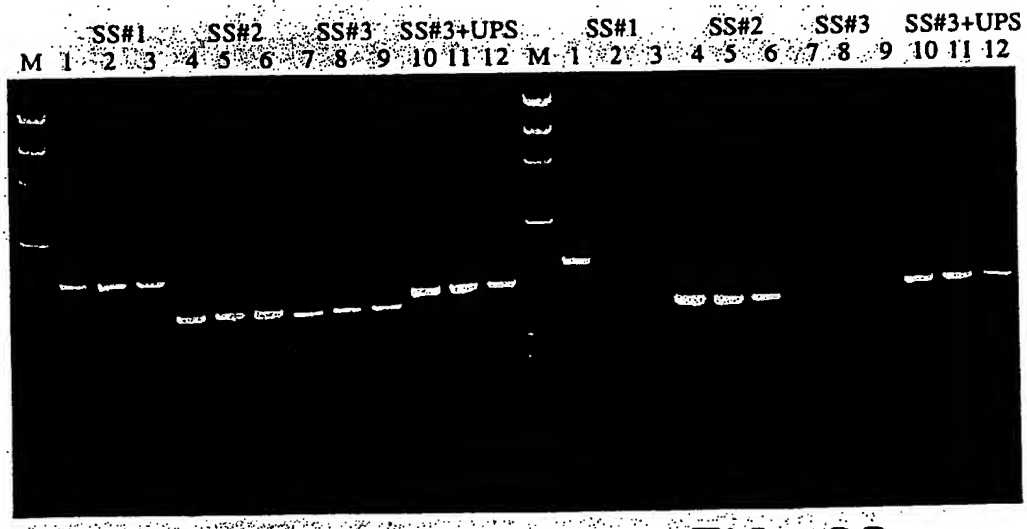

FIG. 2C

FIG. 2D

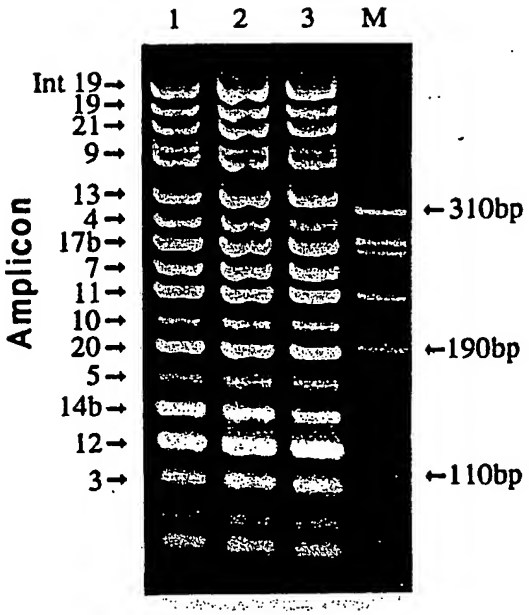
1 2 3 M
Amplicon
Int 19→
19→
21→
9→
13→
4→
17b→
7→
11→
10→
20→
5→
14b→
12→
3→
←310bp
←190bp
←110bp

FIG. 3

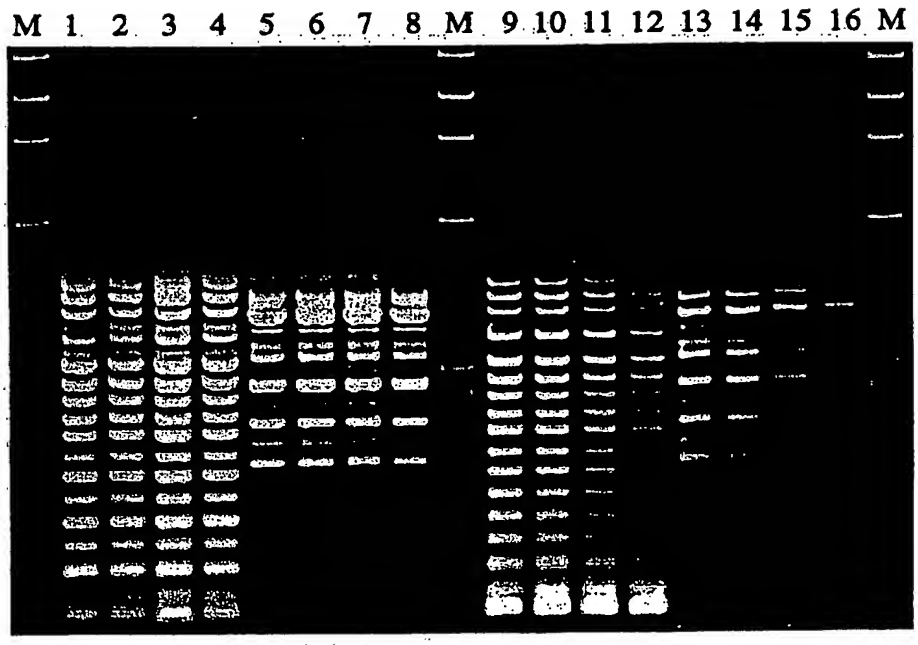
M 1 2 3 4 5 6 7 8 M 9 10 11 12 13 14 15 16 M

FIG. 4

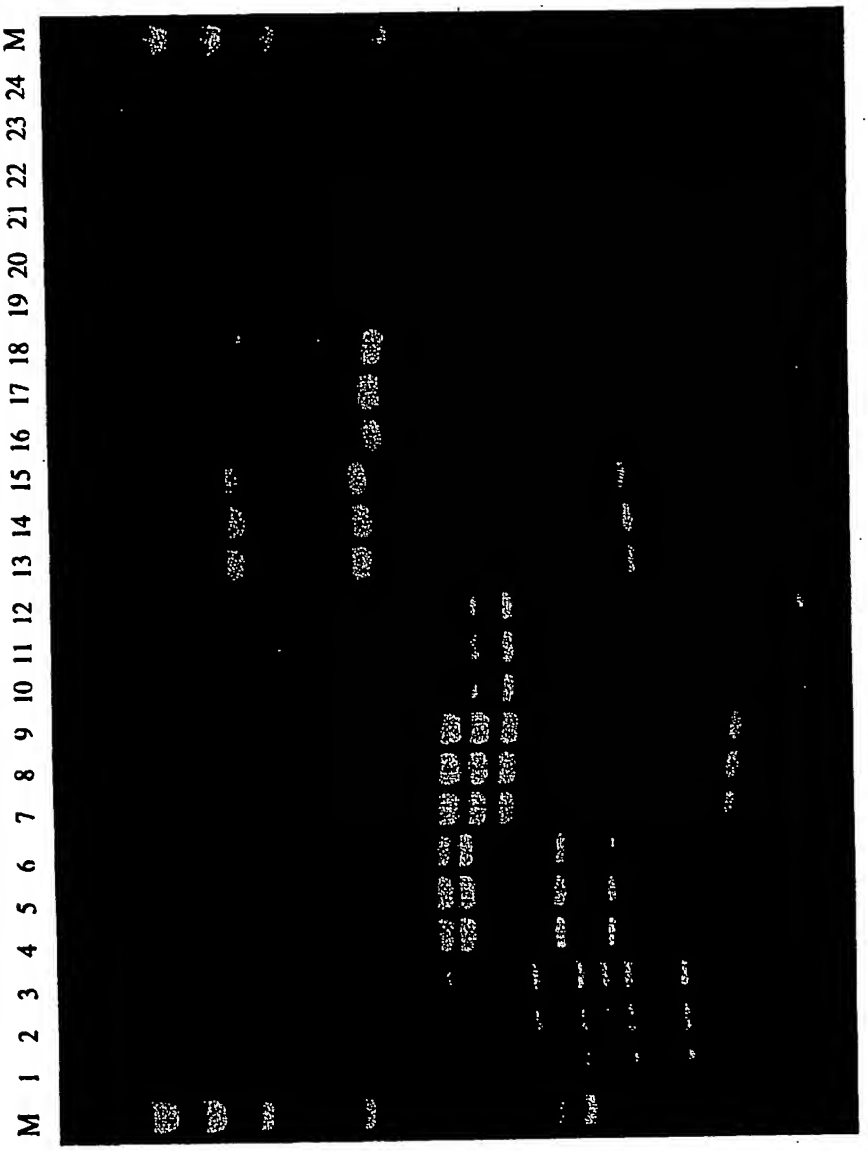

FIG. 5

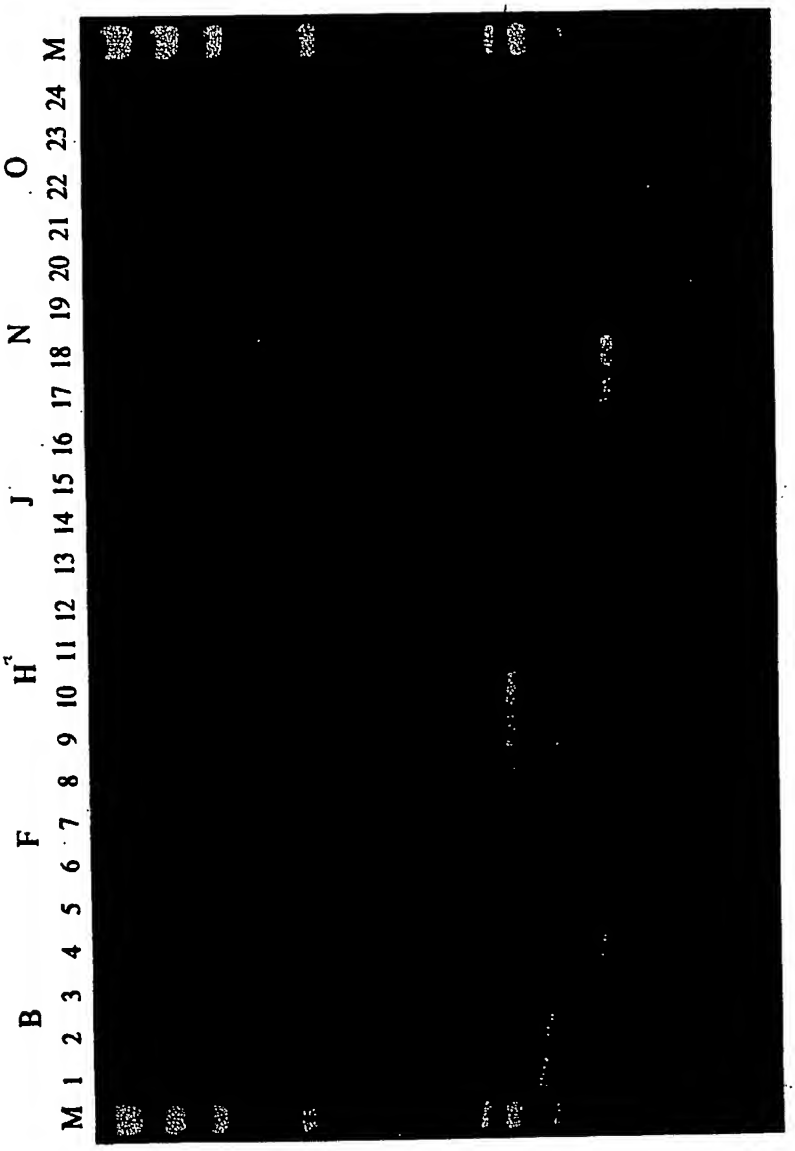

FIG. 6

INTERNATIONAL SEARCH REPORT

Internation· ·pplication No
PCT/US 96/09637

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 628 640 (BECTON, DICKINSON & COMPANY) 14 December 1994 see page 4, line 18 - line 38; claim 10 see page 5, line 56 - page 6, line 10 ---	1,33
A	US,A,5 104 792 (SILVER JONATHAN ET AL) 14 April 1992 see the whole document ---	1-42
A	PCR METHODS AND APPLICATIONS, vol. 3, no. 1, August 1993, CSH LABORATORY PRESS, CSH, NEW YORK, US, pages 77-80, XP000609125 F. WEIGHARDT ET AL.: "A simple procedure for enhancing PCR specificity" cited in the application see the whole document ---	1-42
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 1996	18. 11. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 96/09637

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLINICAL MICRIBIOL. REV., vol. 2, no. 2, April 1989, WASHINGTON, DC, US, pages 217-226, XP000391367 J.C. GUATELLI ET AL.: "Nucleic acid amplification in vitro: Detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection" see page 221, left-hand column, line 3 - line 7 ---	1-42
A	BIOTECHNOLOGY, vol. 9, no. 1, January 1991, NATURE PUBL. CO.,NEW YORK, US, pages 88-89, XP000391625 S.T. JONES AND M.M. BENDIG: "Rapid PCR-cloning of full length mouse immunglobulin variable regions" see the whole document ---	1-42
A	NATURE, vol. 354, 21 November 1991, MACMILLAN JOURNALS LTD., LONDON,UK, pages 204-209, XP002018391 A.J. JEFFREY ET AL.: "Minisatellite repeat coding as a digital approach to DNA typing" see the whole document ---	1-42
A	WO,A,93 18177 (PHILADELPHIA CHILDREN HOSPITAL) 16 September 1993 see the whole document ---	1-42
A	WO,A,93 18178 (PHILADELPHIA CHILDREN HOSPITAL) 16 September 1993 see the whole document ---	1-42
P,X	GENOME RESEARCH, vol. 5, no. 5, December 1995, CSH LABORATORY PRESS, pages 488-493, XP000546034 A.P. SHUBER ET AL.: "A simplified procedure for developing multiplex PCRs" see the whole document -----	1,2, 33-42

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US 96/09637

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EP-A-0628640	14-12-94	US-A-	5422252	06-06-95
		BR-A-	9402174	07-03-95
		JP-A-	8019394	23-01-96
		US-A-	5470723	28-11-95
		US-A-	5561044	01-10-96
US-A-5104792	14-04-92	NONE		
WO-A-9318177	16-09-93	NONE		
WO-A-9318178	16-09-93	NONE		